

# Micronucleus Assay in Lymphocytes as a Tool to Biomonitor Human Exposure to Aneuploidogens and Clastogens

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The analysis of micronuclei (MN) in cultured human lymphocytes is, in principle, able to detect exposure to clastogens and aneuploidogens alike. There is, however, no clear evidence from human biomonitoring studies or animal experiments showing that *in vivo* exposure of resting lymphocytes to an aneuploidogen could actually be expressed as MN in cultured lymphocytes. *In vitro*, a pulse treatment of human lymphocytes with vinblastine, an aneuploidogen, did result in MN induction even if performed before mitogen stimulation, although a much more pronounced effect was obtained in actively dividing lymphocyte cultures. On the other hand, it is probable that a considerable portion of "spontaneous" MN contain whole chromosomes, their contribution increasing with age. It also seems that cytochalasin B, used for the identification of second cell cycle interphase cells in the MN assay, is able to slightly increase the level of MN with whole chromosomes. If MN harboring chromosome fragments represent a minority of the total MN frequency, there may be difficulties in detecting a weak effect in this fraction of MN against the background of MN with whole chromosomes. This would reduce the sensitivity of the assay in detecting clastogens, unless MN with whole chromosomes and chromosome fragments are distinguished from each other. That a problem may exist in sensitivity is suggested by the difficulty in demonstrating MN induction by smoking, an exposure capable of inducing chromosome aberrations. The sensitivity of the lymphocyte MN assay could be increased by detecting kinetochore or centromere in MN, or by automation, allowing more cells to be analyzed.

## Introduction

The analysis of micronuclei (MN) in cultured lymphocytes is increasingly applied as a method to biomonitor human exposure to genotoxic agents, largely because the cytokinesis block (CB) technique (1) has made it possible to identify cells that have divided once in culture (second cycle interphase cells). Most of the MN resulting from *in vivo* exposure to a genotoxin are formed from preexisting lesions when the stimulated lymphocytes divide for the first time *in vitro*.

This paper discusses the applicability of the MN assay for biomonitoring human exposure to genotoxins. The key

questions in this respect are the sensitivity of the assay in detecting exposure to clastogens and aneuploidogens, factors modifying the background level of MN, and the possible influence of cytochalasin B (Cyt-B) used to produce the CB.

## Is Exposure to Aneuploidogens Detected?

It has convincingly been shown in experimental animals that both clastogens and aneuploidogens are able to produce MN in actively dividing tissues (2). Aneuploidogens also induce MN in human lymphocytes when the treatment occurs after mitogen stimulation (3). Although aneuploidogens such as spindle poisons preferentially affect actively dividing cells, exposure of resting lymphocytes (most peripheral lymphocytes are in G<sub>0</sub> stage *in vivo*) might also be expected to induce MN in stimulated lymphocytes if a) the aneuploidogens act upon the G<sub>0</sub> precursors of the mitotic apparatus, b) the concentration of the aneuploidogen present in the blood sample is high enough at the time of the first *in vitro* divisions, or c) MN generated by the exposure *in vivo* are frequent enough to

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be detected against the high background of MN induced "spontaneously" at the first *in vitro* divisions.

Investigations on human exposure to pure aneuploidogens are rare. Two studies describing MN assays after occupational exposure to mercury compounds, known to be aneuploidogenic, have, however, been published. One study of chloroalkali workers exposed to mercury vapor (4), showing correlation of MN frequency with cumulative exposure index and with the number of blood mercury peaks, was difficult to interpret because Cyt-B was not employed. The other study, which used the CB method and described increased MN frequencies among explosives workers exposed to mercury fulminate, might have reflected clastogenic exposure as well because the workers also had elevated levels of chromosome aberrations (5).

A simple way to study the question of whether MN can be induced by aneuploidogen exposure of resting lymphocytes is to treat lymphocytes with an aneuploidogen before mitogen stimulation. Results from this kind of experiment with vinblastine sulfate are shown in Figure 1. MN induction was observed with all of the treatment schedules, but there were enormous differences in the response. Treatments started when phytohemagglutinin (PHA) had been present for 24 hr and continued for 72 or 48 hr until cell harvest, providing good possibilities for the drug to interact with dividing cells. This treatment resulted in an efficient induction of MN at such low concentrations of vinblastine as 1 or 2.5 ng/mL, respectively, whereas a 24-hr pulse treatment between 24 and 48 hr (with less mitoses available) of 72-hr cultures required 40 ng/mL of vinblastine for a positive response. It is also evident from

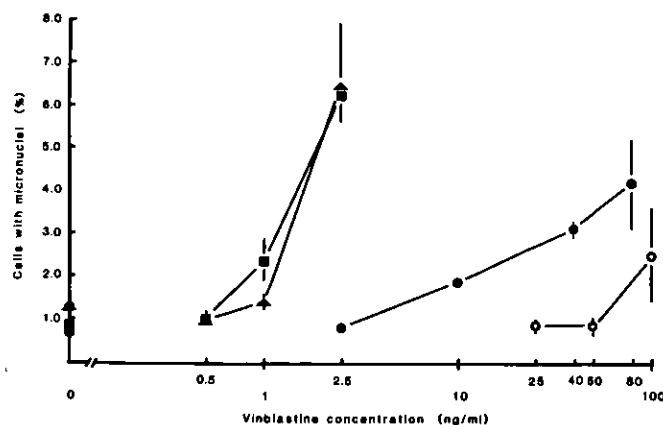


FIGURE 1. Effect of vinblastine sulfate on micronucleated binucleate lymphocytes in whole-blood cultures of two male blood donors. The symbols represent means; error bars show individual results from the two donors. (■) 72-hr treatment from hour 24 of culture until harvest; (▲) 48-hr treatment from hour 24 of culture until harvest; (●) 24-hr pulse treatment between hour 24 and 48 of a 72-hr culture; (○) 24-hr pulse treatment without phytohemagglutinin (PHA; present in other cultures from the start), followed by 72-hr incubation with PHA. At the end of the 24-hr pulse treatment, the cells were washed twice with phosphate-buffered saline. Cytochalasin B was added at 44 hr (in the pulse treatment of stimulated lymphocytes at 48 hr) after culture initiation or PHA addition; 1000 cells were scored per treatment from air-dried May-Grünwald-Giemsa-stained slides.

Figure 1 that the 24-hr pulse treatment of nondividing cells, performed before mitogen stimulation, was the least effective, yielding a positive result (a 3.7-fold elevation above the control level) only at the highest concentration available for analysis (100 ng/mL; 250 ng/mL was toxic). It was not possible to tell whether this response was due to an effect induced in the resting stage and retained until mitosis or to residual vinblastine left in the cells or in the culture medium, despite the wash. Nevertheless, elevation of lymphocyte MN levels in a human biomonitoring study cannot be expected to result from *in vivo* exposure to some unestablished or unknown aneuploidogenic agent before it has been shown experimentally or by human studies that an *in vivo* aneuploidogen exposure is able to influence MN levels in cultured lymphocytes.

## Is Exposure to Clastogens Detected?

Studies on patients exposed to ionizing radiation and chemotherapy have clearly shown that an *in vivo* exposure to clastogens can result in MN induction in stimulated blood lymphocytes (6-10). Experience with lower exposure levels such as encountered in occupational chemical exposure has been less encouraging.

Since the first human biomonitoring study (11,12) using Cyt-B—which did show elevated MN levels among cyclophosphamide process workers and oncology nurses exposed to cytostatics—biomonitoring studies with a positive MN outcome from exposures to chemical genotoxins (other than cytostatics in cancer therapy) have been rare. In Finland, investigations of exposure to styrene in the reinforced plastics industry (13,14), power-line workers exposed to electric and magnetic fields (15), and formaldehyde-exposed industry workers (Table 1) have all yielded a negative outcome for MN. A tentative positive finding was recorded in one study comparing lymphocyte MN levels among hospital pharmacists before and after 1 year of handling of cytostatics, although the difference to a matched control group was not significant (Table 1). Biomonitoring studies from other countries have found no deviation from control MN frequencies in tannery workers (16), ethylene oxide-exposed sanitary workers (17), and human volunteers taking the clastogenic analgesic paracetamol (18).

With so few studies available, the negative outcomes could reflect the fact that the exposures studied were not the best choices for evaluating the lymphocyte MN assay. Nevertheless, in several of the investigations mentioned above (15-18), some other parameter of genotoxic exposure (e.g. MN in buccal mucosa in our study of formaldehyde exposure; data not published) gave positive results.

Another exposure, which usually produces elevations in chromosome aberrations and sister chromatid exchanges, but not in MN, is smoking (4,5,11-13,16). Au et al. (19) found a slight but nonsignificant elevation of MN among smokers. Although Tomanin et al. (20) were able to show a statistically significant difference in MN levels between smokers and nonsmokers, the overall impression that one gets from the few studies available is that the CB MN assay does not detect smoking as efficiently as the analysis of chromosome aberrations does.

**Table 1. Micronuclei (MN) in binucleated lymphocytes in whole-blood cultures of formaldehyde-exposed workers (plywood, fiberglass, and impregnation plants) and hospital pharmacists exposed to cytostatics.<sup>a</sup>**

Studied group	No. of subjects	No. of men	No. of smokers	Mean age, years	Mean % (SD) cells with MN
Formaldehyde exposed	22	16	6	40	2.5 (0.8)
Controls	22	16	6	40	3.0 (1.0)
Pharmacists					
1990	7	0	0	39	3.0 (1.1)
1991	7	0	0	40	3.7 (1.0)*
Controls					
1990	7	0	0	39	3.0 (0.2)
1991	7	0	0	40	3.3 (0.8)

<sup>a</sup>Formaldehyde-exposed workers: 1000 cells scored/person, acridine orange staining; hospital pharmacists: 500 cells scored/sample, May-Grünwald-Giemsa staining. Both studies had a control group matched for sex, age, and smoking habits. For hospital pharmacists, sampling was performed before and after 1 year of exposure.

\* $p < 0.05$ , 1991 versus 1990 (two-tailed *t*-test for paired observations).

A very interesting finding in this context is that of Larramendy and Knuutila (21), which suggests that some immunologically classified subsets of cultured human lymphocytes, such as pan B cells (CD20+CD22) and, especially, suppressor/cytotoxic T8 lymphocytes (CD8) are more sensitive than others to MN induction by smoking. For the T8 lymphocytes the difference between smokers and nonsmokers in MN levels was almost 15-fold. Cyt-B was not used in the MN evaluation, but the analysis of mitotic indices (at 72 hr) indicated no differences in mitotic activity between smokers and nonsmokers.

### Micronuclei Containing Whole Chromosomes: Effect of Age and Cytochalasin B

Several studies (4,6,8,11,12,14,16,19) have demonstrated that age affects the MN frequency of cultured human lymphocytes, the effect being steeper for women than for men. Lymphocyte MN appear to contain whole chromosomes more often among the elderly (>65 years) than among young people (20–35 years), as indicated by the frequency of MN staining positively with antikinetochore antibodies (50% and 42%, respectively), although some age-related MN might harbor whole chromosomes with inactivated centromeres and lack kinetochore staining (6,22).

Our unpublished findings with cultured lymphocytes of three male subjects (aged 32–39 years) indicate that the majority of MN contain a centromere and that MN are more frequently kinetochore positive in Cyt-B-induced (3  $\mu\text{g/mL}$ ) binucleate cells (mean 84%) than in mononucleate cells of cultures not containing Cyt-B (mean 63%). This would suggest that Cyt-B induces MN with whole chromosomes.

In fact, it has quite conclusively been shown that Cyt-B induces MN in human lymphocytes (23). High MN frequencies are, however, restricted to multinucleate (cells with more than two nuclei) lymphocytes, which are formed

when the binucleate cells further divide. The MN are probably the result of disorganized multipolar divisions and, because multipolar anaphases in Cyt-B-treated human lymphocyte cultures have a high level of lagging chromosomes, contain whole chromosomes (23).

The important question is, of course, does Cyt-B affect MN in binucleate cells. Fenech and Morley (1) reported that there was no difference in MN frequencies between binucleate cells produced by Cyt-B and mononucleate cells not treated with Cyt-B. In accordance with the results of Prosser et al. (24), we observed no dose response in MN frequencies in binucleate cells at different concentrations of Cyt-B (23). On the other hand, we found no dose dependency for MN in multinucleate cells either, although they contained some 9–21 times higher basic MN frequencies than the binucleate cells. Dose response may not be expected when the analysis is restricted to cells that are identified as being affected (i.e., multinucleated or binucleated) by Cyt-B.

Our experiments did suggest a dose-dependent increase in lagging chromosomes, although only the regression was significant, in bipolar anaphases of Cyt-B-treated human lymphocyte cultures (Table 2). As it is known that a decreasing portion of dividing cells are able to escape the CB with increasing concentrations of Cyt-B (25), the dose response could be explained if one assumes that the bipolar divisions committed to become binucleated have an increased tendency to develop lagging chromatids. Table 2 may also suggest an influence of Cyt-B on the frequency of anaphase bridges, although there is no dependency on Cyt-B concentration.

It is not yet clear what percentage of the MN seen in binucleated cells is due to Cyt-B, although the possible effect cannot be nearly as dramatic as seen in multinucleate cells produced by Cyt-B. In any case, unless kinetochores (2,3,22,26) or centromeres (27,28) (Fig. 2) in MN are labeled, there may be difficulties in detecting even a doubling in MN harboring chromosome fragments against the background of MN with whole chromosomes, which are uninformative with respect to clastogenic exposure.

Table 2. Effect of a 28-hr treatment (from hour 44 until harvest) with cytochalasin B (Cyt-B) on the frequency of bipolar anaphases with aberrations.<sup>a</sup>

Cyt-B concentration, μg/mL	No. of anaphases studied	Anaphases with aberrations, mean % (SD)		
		Lagging chromatin*	Bridges	Total
0	587	1.5 (1.5)	0.3 (0.3)	1.9 (1.8)
1.5	532	2.2 (1.0)	1.5 (1.3)†	3.2 (1.8)
3.0	142	2.9 (1.3)	0.7 (1.3)	2.9 (1.3)
6.0	78	3.4 (3.4)	0.0 (0.0)	4.5 (5.1) <sup>b</sup>
12.0	92	4.2 (0.6)	1.3 (2.3)	4.2 (0.6)

<sup>a</sup>Means from the isolated lymphocyte cultures of three male donors and standard deviations are shown (26).

<sup>b</sup>One anaphase with a disrupted pole included.

\*Linear regression significant ( $p < 0.01$ , two-tailed  $t$ -test);  $r = 0.95$ .

† $p < 0.05$ , Fisher's exact probability test (1-tailed).

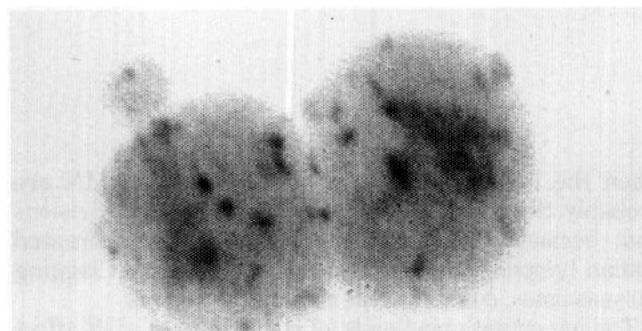


FIGURE 2. A binucleated human lymphocyte with a micronucleus showing a centromeric signal. *In situ* hybridization of the aliphoid DNA oligomer probe SO- $\alpha$ AllCen (28) with the sequence GTTTTGAAAC<sup>10</sup>. ACTCTTTTGTG<sup>20</sup>TAGAATCTGC<sup>30</sup>; digoxigenin label and immunoperoxidase/diaminobenzidine detection; counterstaining with Wright's stain.

## Improving the Sensitivity of Human Lymphocyte Micronucleus Assay

It is obvious that the sensitivity of the MN assay in monitoring human exposure to genotoxins could be increased in several different ways. In revealing the specific effects of clastogens, considerable improvement could be achieved by including only those MN that do not have a kinetochore or a centromere (3,22,26). If *in vivo* exposure to aneuploidogens turns out to be detectable by the MN assay, the same techniques could be used to distinguish the influence of both the clastogenic and aneuploidogenic components of an *in vivo* exposure. Kinetochore/centromere detection would also remove the possible problem of Cyt-B inducing MN harboring whole chromosomes. On the other hand, the CB method could be replaced by other assays for identifying second cycle interphases. We have developed one such alternative, based on the use of pulse labeling with 5-bromodeoxyuridine followed by label detection by antibromodeoxyuridine antibody (29). If T8 lymphocytes really are much more sensitive to smoking-induced MN than other lymphocyte subpopulations (21), MN analysis could possibly be restricted to these lymphocytes alone also in other exposures.

Finally, as the resolution of the analysis of such relatively rare events as MN could be enhanced by increasing the number of cells scored per sample, the development of automated approaches to MN analysis would also much improve the sensitivity of the assay (2).

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## REFERENCES

1. Fenech, M., and Morley, A. A. Measurement of micronuclei in lymphocytes. *Mutat. Res.* 147: 29-36 (1985).
2. Heddle, J. A., Cimino, M. C., Hayashi, M., Romagna, F., Shelby, M. D., Tucker, J. D., Vanparys, Ph., and MacGregor, J. T. Micronuclei as an index of cytogenetic damage: past, present, and future. *Environ. Mol. Mutagen.* 18: 277-291 (1991).
3. Eastmond, D. A., and Tucker, J. D. Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. *Environ. Mol. Mutagen.* 13: 34-43 (1989).
4. Barregård, L., Högstedt, B., Schütz, A., Karlsson, A., Sällsten, G., Thiringer, G. Effects of occupational exposure to mercury vapor on lymphocyte micronuclei. *Scand. J. Work Environ. Health* 17: 263-268 (1991).
5. Anwar, W. A., and Gabal, M. S. Cytogenetic study in workers occupationally exposed to mercury fulminate. *Mutagenesis* 6: 189-192 (1991).
6. Fenech, M. Optimisation of micronucleus assays for biological dosimetry. In: *New Horizons in Biological Dosimetry* (B. L. Gledhill and F. Mauro, Eds.), Wiley-Liss, New York, 1991, pp. 373-386.
7. Fenech, M., Denham, J., Francis, W., and Morley, A. Micronuclei in cytokinesis-blocked lymphocytes of cancer patients following fractionated partial-body radiotherapy. *Int. J. Radiat. Biol.* 57: 373-383 (1990).
8. Migliore, L., Guidotti, P., Favre, C., Nardi, M., Sessa, M. R., and Brunori, E. Micronuclei in lymphocytes of young patients under antileukemic therapy. *Mutat. Res.* 263: 243-248 (1991).
9. Sarto, F., Tomanin, R., Giancomelli, L., Canova, A., Raimondi, F., Ghiotto, C., and Fiorentino, M. V. Evaluation of chromosomal aberrations in lymphocytes and micronuclei in lymphocytes, oral mucosa and hair root cells of patients under antitlastic therapy. *Mutat. Res.* 228: 157-169 (1990).
10. Sinúes, B., Nuñez, E., Bernal, M. L., Alcalá, A., Saenz, M. A., and Conde, B. Micronucleus assay in biomonitoring of patients undergoing excretory urography with diatrizoate and ioxaglate. *Mutat. Res.* 260: 337-342 (1991).
11. Yager, J. W., Sorsa, M., Selvin, S. Micronuclei in cytokinesis-blocked lymphocytes as an index of occupational exposure to alkylating cytostatic drugs. In: *Methods for Detecting DNA Damaging Agents in Humans: Applications in Cancer Epidemiology and Prevention* (H. Bartsch, K. Hemminki, and I. K. O'Neill, Eds.), IARC Scientific

- Publication No. 89, International Agency for Research on Cancer, Lyon, 1988, pp. 213-216.
12. Sorsa, M., Pyy, L., Salomaa, S., Nylund, L., and Yager, Y. W. Biological and environmental monitoring of occupational exposure to cyclophosphamide in industry and hospitals. *Mutat. Res.* 204: 465-479 (1988).
  13. Mäki-Paakkanen, J., Walles, S., Osterman-Golkar, S., and Norppa, H. Single-strand breaks, chromosome aberrations, sister chromatid exchanges, and micronuclei in blood lymphocytes of workers exposed to styrene during the production of reinforced plastics. *Environ. Mol. Mutagen.* 17: 27-31 (1991).
  14. Sorsa, M., Anttila, A., Järventaus, H., Kubiak, R., Norppa, H., Nylander, L., Pekari, K., Pfäffli, P., and Vainio, H. Styrene revisited - exposure assessment and risk estimation in reinforced plastics industry. In: *New Horizons in Biological Dosimetry* (B. L. Gledhill, and F. Mauro, Eds.), Wiley-Liss, New York, 1991, pp. 187-195.
  15. Paile, W., Salomaa, S., Jokela, K., Koivistoinen, A., Norppa, H., Sorsa, M., Järventaus, H., Valjus, J., Järvinen, P., and Kajander, J. Cytogenetic effects of 50-Hz EM fields - occupational and *in vitro* studies. In: *Abstracts of the Thirteenth Annual Meeting of the Bioelectromagnetics Society*, Salt Lake City, Utah, June 23-27, 1991.
  16. Migliore, L., Parrini, M., Sbrana, I., Biagini, C., Battaglia, A., and Loprieno, N. Micronucleated lymphocytes in people occupationally exposed to potential environmental contaminants: the age effect. *Mutat. Res.* 256: 13-20 (1991).
  17. Sarto, F., Törnqvist, M. A., Tomanin, R., Bartolucci, G. B., Osterman-Golkar, S. M., and Ehrenberg, L. Studies of biological and chemical monitoring of low-level exposure to ethylene oxide. *Scand. J. Work Environ. Health* 17: 60-64 (1991).
  18. Kočíšová, J., and Šrám, R. J. Mutagenicity studies on paracetamol in human volunteers III. Cytokinesis block micronucleus method. *Mutat. Res.* 244: 27-30 (1990).
  19. Au, W. W., Walker, D. M., Ward, J. B., Jr., Whorton, E., Legator, M. S., Singh, V. Factors contributing to chromosome damage in lymphocytes of cigarette smokers. *Mutat. Res.* 260: 137-144 (1991).
  20. Tomanin, R., Ballarin, C., Nardini, B., Mastrangelo, G., and Sarto, F. Influence of smoking habit on the frequency of micronuclei in human lymphocytes by the cytokinesis block method. *Mutagenesis* 6: 123-126 (1991).
  21. Larramendy, M. L., and Knuutila, S. Increased frequency of micronuclei in B and T8 lymphocytes from smokers. *Mutat. Res.* 259: 189-195 (1991).
  22. Fenech, M., and Morley, A. A. Kinetochore detection in micronuclei: an alternative method for measuring chromosome loss. *Mutagenesis* 4: 98-104 (1989).
  23. Lindholm, C., Norppa, H., Hayashi, M., and Sorsa, M. Induction of micronuclei and anaphase aberrations by cytochalasin B in human lymphocyte cultures. *Mutat. Res.* 260: 369-375 (1991).
  24. Prosser, J. S., Moquet, J. E., Lloyd, D. C., and Edwards, A. A. Radiation induction of micronuclei in human lymphocytes. *Mutat. Res.* 199: 37-45 (1988).
  25. Littlefield, L. G., Sayer, A. M., and Frome, E. L. Comparison of dose-response parameters for radiation-induced acentric fragments and micronuclei observed in cytokinesis-arrested lymphocytes. *Mutagenesis* 4: 265-270 (1989).
  26. Thomson, E. J., and Perry, P. E. The identification of micronucleated chromosomes: a possible assay for aneuploidy. *Mutagenesis* 3: 415-418 (1988).
  27. Becker, P., Scherthan, H., and Zanki, H. Use of a centromere-specific DNA probe (P82H) in nonisotopic *in situ* hybridization for classification of micronuclei. *Genes Chrom. Cancer* 2: 59-62 (1990).
  28. Meyne, J., Littlefield, L. G., and Moyzis, R. K. Labeling of human centromeres using an alphoid DNA consensus sequence: application to the scoring of chromosome aberrations. *Mutat. Res.* 226: 75-79 (1989).
  29. Autio, K., Norppa, H., Hayashi, M., Mäki-Paakkanen, J., and Sorsa, M. Human lymphocyte micronucleus assay using antibromodeoxyuridine antibody instead of cytochalasin. *Mutat. Res.* 252: 217-218 (1991).